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ABSTRACT

The apical membrane of high electrical resistance epithelia, which is selectively permeable to sodium ions, plays an essential role in the maintenance of salt balance. Sodium entry from the apical fluid into the cells is mediated by amiloride-blockable Na^+-specific channels. The channel protein, purified from both amphibian and mammalian sources, is composed of several subunits, only one of which, the 150 kDa polypeptide, specifically binds the Na^+ transport inhibitor, amiloride. The goal of the present study was to investigate whether the isolated amiloride-binding subunit of the channel could conduct Na^+ ions. The patch-clamp technique was used to study the 150 kDa polypeptide incorporated into a lipid bilayer formed on the tip of a glass pipet. Unitary conductance jumps averaged 4.8 pS at 100 mM Na_2HPO_4. Open times ranged from 24 msec to several seconds. The channel spent most of the time in the closed state. Channel conductance and gating were independent of voltage between -60 and +100 mV. Amiloride (0.1 μM) decreased the channel's mean open time by 98%. We conclude that the 150 kDa subunit of the amiloride-blockable sodium channel conducts current and may be sufficient for the sodium transport function of the whole channel.

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Single-channel behavior of a purified epithelial Na^+ channel subunit that binds amiloride

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Sariban-Sohraby, Sarah, Maurice Abramow, and Richard S. Fisher. Single-channel behavior of a purified epithelial Na^+ channel subunit that binds amiloride. *Am. J. Physiol.* 263 (*Cell Physiol.* 32): C1111-C1117, 1992.—The apical membrane of high electrical resistance epithelia, which is selectively permeable to Na^+, plays an essential role in the maintenance of salt balance. Na^+ entry from the apical fluid into the cells is mediated by amiloride-blockable Na^+-specific channels. The channel protein, purified from both amphibian and mammalian sources, is composed of several subunits, only one of which, the 150-kDa polypeptide, specifically binds the Na^+ transport inhibitor amiloride. The goal of the present study was to investigate whether the isolated amiloride-binding subunit of the channel could conduct Na^+. The patch-clamp technique was used to study the 150-kDa polypeptide incorporated into a lipid bilayer formed on the tip of a glass pipette. Unitary conductance jumps averaged 4.8 pS at 100 mM Na_2HPO_4. Open times ranged from 24 ms to several seconds. The channel spent most of the time in the closed state. Channel conductance and gating were independent of voltage between −60 and +100 mV. Amiloride (0.1 μM) decreased the mean open time of the channel by 98%. We conclude that the 150-kDa subunit of the amiloride-blockable Na^+ channel conducts current and may be sufficient for the Na^+ transport function of the whole channel.

patch clamp; sodium transport; protein purification; electrophysiology; ion channels; beef papilla; A6 cultured cells; epithelium

ENTRY OF SODIUM INTO Na^+-reabsorbing epithelial cells is mediated by selective channels located in the apical plasma membrane of these cells (14). The native apical epithelial Na^+-conducting protein is composed of five (or six) subunits with molecular masses that range from 315 to 35 kDa (3, 11). The channels are reversibly inhibited with high affinity by amiloride and its analogues that have served as useful tools for isolation and purification of the channel protein. At every step of the channel purification, the Na^+ transport function is maintained (4, 23, 25), but the transport properties of the individual subunits have not yet been examined.

It was previously shown by using the amiloride analogue [^{3}H]methylbromoamiloride as a photoaffinity label (12), that the binding site of the blocker is located on the 150-kDa polypeptide (3). We therefore assessed the Na^+ transport function of this subunit after its isolation and incorporation into a lipid bilayer. The electrical properties of the isolated 150-kDa subunit reconstituted into membrane patches from giant liposomes were evaluated using the patch-clamp technique (16, 27).

MATERIALS AND METHODS

Preparation of Na^+ channel subunits. Na^+ channels were prepared as described previously (2, 23). In brief, bovine kidney papillae plasma membrane fraction was solubilized with the detergent 3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate (CHAPS) and used as a source material for further purification of the apical Na^+ channel. This partially purified fraction retains amiloride-blockable ^{22}Na uptake into lipid vesicles (23). Samples then were subjected to affinity chromatography on wheat germ lectin columns and gel filtration size-exclusion high-performance liquid chromatography (HPLC; Zorbax GF450; Du Pont, Belgium), which yielded the native 740-kDa channel protein as described previously (2). This protein complex was reduced with 10 mM dithiothreitol for 1 h, and separation of the Na^+ channel subunits was achieved on a second gel filtration HPLC column (Zorbax GF 250) using a 100 mM Na_2HPO_4 (pH 7.1) elution buffer. Individual subunit peaks were collected virtually free of detergent, and the sample volume of each peak was decreased in microconcentrators (Centricon 10; Amicon, Beverly, MA). Each peak was then rechromatographed separately on the Zorbax GF250 column, and collected samples were frozen at −80C until reincorporated into liposomes.

Protein radioiodination. The 150-kDa HPLC peak (10–15 ng in 500 μl of 100 mM Na_2HPO_4, pH 7.0) was iodinated using the chloramine-T method. One millicurie $Na^{125}I$ (New England Nuclear) was added to the sample on ice. At 0, 2.5, 5, and 7.5 min, 10 μl chloramine-T (1 mg/ml in water) were added to the reaction mixture. After 10 min, the reaction was stopped by the addition of 100 μl sodium metabisulfite (1 mg/ml in water). Labeled protein was separated from free iodide by three passages on Sephadex G-25 to G-150 columns.

Gel electrophoresis. Polyacrylamide gel electrophoresis was carried out in a mini-protean II slab gel apparatus (Bio-Rad). Acrylamide concentration was 9%, and no stacking gel was used. Samples were prepared for electrophoresis in 50 mM tris-(hydroxymethyl)aminomethane (Tris)·HCl buffer (pH 6.8) containing 2.5% sodium dodecyl sulfate (SDS) and 10 mM dithiothreitol. Electrophoresis was performed at a constant voltage of 200 V for 45 min. Radioiodinated bands were visualized by exposure of the dried gels to Hyperfilm-MP (Amersham) at −80C. Films were developed by autoprocessing.

Preparation of freeze-thaw liposomes. The small quantity of purified subunits precluded standard preparation of liposomes in which lipids and protein are mixed at the appropriate ratio (10, 27). Instead, we attempted incorporation of the subunits into a lipid bilayer membrane preformed at the tip of a glass micropipette. Blank freeze-thaw liposomes (FTL) were prepared from a mixture of phosphatidylcholine-phosphatidylethanolamine-phosphatidylserine-cholesterol (5:5:2:2 by weight;

Sigma Chemical) that was hydrated for 30 min at 37°C with an aqueous solution (150 mM KCl, 1 mM $CaCl_2$, pH 7.5). The lipid mixture was sonicated on ice, yielding small unilamellar vesicles, divided into 10-μl samples in plastic vials, and then dropped into liquid nitrogen where they were stored for at least 24 h (10). For use, a sample was thawed at room temperature, pipetted into a 0.5-ml bath containing aqueous salt solution, and gently swirled to disperse the lipids. After 30 min, some of the lipid sample settled to the bottom of the bath (a glass slide), mostly forming multilamellar liposomes and various irregular clumps of lipid. At this time, fresh aqueous solution was perfused into the bath to remove floating debris. Some material remained attached to the glass slide at the bottom of the chamber, and, using transmission light microscopy to visualize attached liposomes, we attempted to examine the electrical properties of isolated patches of the lipid material.

Electrical measurements. The patch-clamp technique was used to measure the electrical properties of the liposomes (16, 27). Glass pipettes were manufactured with a two-stage vertical microelectrode puller and then fire polished. The tip was filled by dipping in a solution of 100 mM Na_2HPO_4, and, after backfilling, electrode resistance averaged ~10 MΩ. Single-channel currents were measured with a patch-clamp amplifier (model L/M-EPC5; List Medical, FRG). The liposome bath chamber consisted of a glass slide with a Lucite block (thickness 3 cm) glued to the upper surface of the slide. The Lucite block contained a 1-cm diameter hole, which formed the bath, and ports for perfusing solution. An additional hole (5 mm) was drilled in the Lucite and served as a reservoir to hold lipid-free bath solution for patch transfers (*transfers 1* and *3*; see RESULTS). A small conical vial containing the subunit solution was placed near these wells to facilitate *transfer 2* of the electrode tip and bilayer. An Ag-AgCl reference electrode was dipped in the bath. Patch-electrode current was amplified and filtered at 300 Hz, resulting in <0.2 pA electrical noise. Analog data were stored with an FM tape recorder, which were later digitized and analyzed by computer with software written by G. Droogmans (Katholieke Universiteit, Leuven, Belgium).

Formation of high-resistance patches of FTL. The liposomes that settled to the bottom of the bath varied in size and shape. Some structures were very large (>100-μm diameter); the membrane thickness varied substantially from several microns to very thin. Attempts to patch thick membranes or lipid clumps always yielded plugged electrode tips. Sometimes we could locate a few large liposomes (>20 μm) with membranes that were barely visible and that most likely consisted of a single lipid bilayer (19). Stable high-resistance patches were only obtained from these structures. The patch-clamp electrode was advanced through the bath with a micromanipulator, and a small amount of pressure on the electrode solution was maintained to force fluid out of the electrode tip, which prevented plugging by floating clumps of lipid (27). Usually, an indentation of the target FTL was observed when the tip was within a few microns of the liposome. Gigaohm seals of FTL often were obtained by simply releasing the outward pressure, i.e., with little or no suction on the electrode. Low-resistance patches (<15 GΩ) of blank liposomes were electrically unstable and sometimes yielded apparent channel activity immediately after seal formation (8). In contrast, high-resistance seals (>25 GΩ) were mechanically and electrically more stable occasionally for long periods of time (<3 h). After seal formation, the electrode was briefly exposed to air, and the patch was moved to a liposome-free bath (*transfer 1*). The success rate for maintaining high-resistance seals through *transfer 1* was ~5%.

Data analysis. We assumed channel openings were random and independent. Because of the complexity of incorporating subunits into liposome patches, we were unable to obtain a sufficient number of events to provide a statistically significant detailed kinetic analysis (i.e., from open and closed time histograms). However, similar to the analysis by Light et al. (13), we determined mean open time (MOT) as the arithmetic average of the dwell time of the channel in the open state. The MOT is defined in the present study as the sum of the channel open times divided by the number of events. The channel open probability (P_o) is defined as the sum of the open times divided by the total length of the observation period. The beginning of the observation period was delineated by the first transition from the closed to the open state; the end was marked by the last open-to-closed transition before loss of the stable preparation. The percent open time is equal to $100 \times P_o$. Current amplitude and duration measurements were performed for each discrete event using cursors positioned by eye on digitized current traces.

RESULTS

The use of standard artificial bilayer techniques for functional characterization of the Na^+ channel subunit was rejected because of the minute quantities of sample available. The feasibility of utilizing the patch-clamp technique with a detergent-solubilized sample was assessed first on the CHAPS-solubilized partially purified plasma membrane fraction (see MATERIALS AND METHODS). This crude membrane fraction was included in the hydration step during the preparation of FTL. Single-channel activity was observed with unitary conductance jumps of ~10 pS in 150 mM NaCl (Fig. 1). Open times ranged from 80 ms to several seconds, and amiloride (10^{-7} M) induced flickering of the channel between the open and blocked states (data not shown). Because this behavior is consistent with previous observations of apical Na^+ channels studied in situ by patch-clamp technique in A6 toad kidney cultured cells (5), toad urinary bladder (6), and rat collecting tubule (18), we proceeded with the study of the purified subunits of the Na^+ channel protein.

Separation of the subunits was achieved by two consecutive HPLC runs on a GF-250 column. In the first run, dithiothreitol-reduced protein was resolved into several peaks that were collected separately (Fig. 2, *top trace*). Then each peak was rerun to assure higher homogeneity of the samples as shown in the bottom trace of Fig. 2 for the 150-kDa polypeptide. The results from polyacrylamide gel electrophoresis (PAGE) separation of the 150-kDa fraction from each of the two consecutive HPLC runs are shown in Fig. 3. Figure 3, *left lane*, corresponds to the 150-kDa fraction collected after the first HPLC separation. It shows contamination with the 70-kDa subunit of the channel, which disappears during the second HPLC separation. There are two polypeptides of relative molecular masses (M_r) 40 and 31 kDa, which remain even after the second HPLC run. The 40-kDa component could represent either fetuin, which copurifies with the channel (Sariban-Sohraby, unpublished observations) or the $G\alpha_{1-3}$ protein (1). The 31-kDa component may represent a channel subunit that is occasionally but not systematically resolved (3). Thus the data shown in Fig. 3 indicate that the 150-kDa fraction of the second HPLC separation is substantially enriched with the 150-kDa polypeptide (see DISCUSSION).

The small amount of purified 150-kDa subunit available (<1 pmol/40 kidneys) precluded its incorporation into FTL during the hydration step (see MATERIALS AND

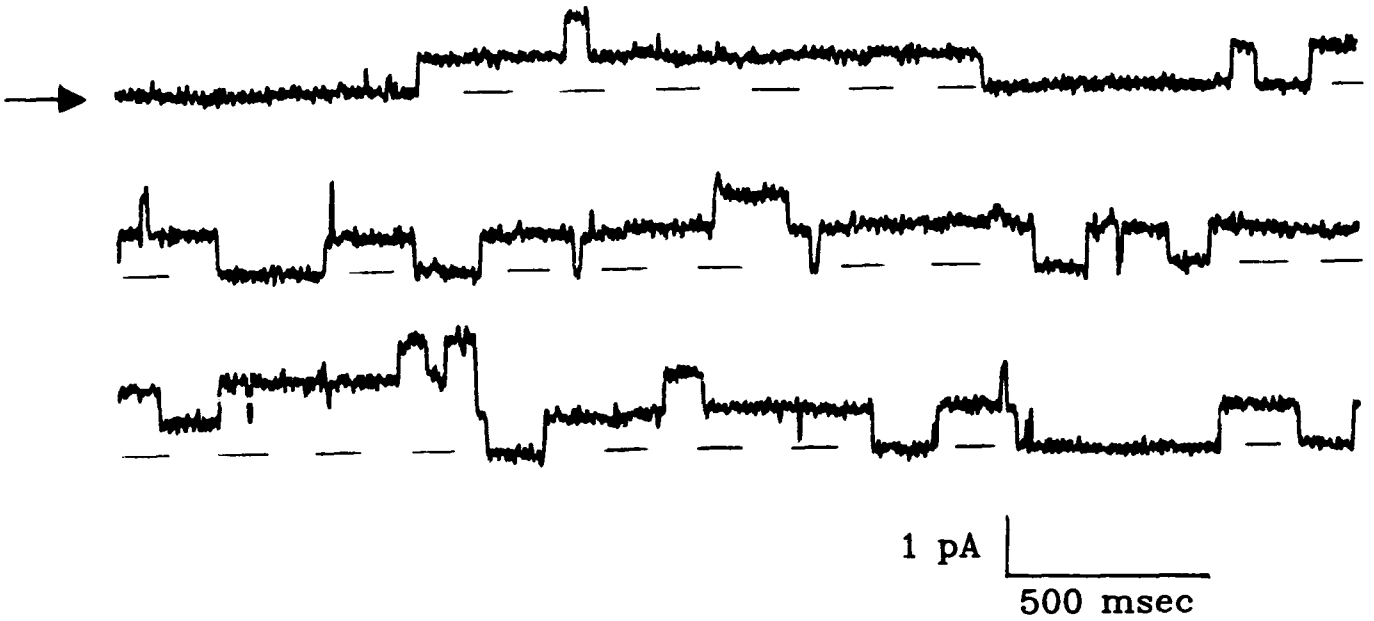

Fig. 1. Electrical activity from excised patch of freeze-thaw liposomes prepared with crude membrane fraction (i.e., whole Na^+ channel). Single-channel conductance was 10 pS when bathed in 150 mM NaCl; pipette voltage ($V_{pipette}$) = +60 mV. Arrows and dashed lines indicate current measured from quiescent membrane.

METHODS). Therefore blank liposomes were patched ($n \approx$ 3,800) and yielded an apparent bilayer patch with high electrical resistance ~15% of the time (n = 558). If the integrity of the high-resistance patch was maintained after moving the electrode through air to the lipid-free bath (*transfer 1*, see above; n = 197), the electrode tip was again removed from the bath and then dipped into a conical vial containing <0.1 pmol purified 150-kDa subunit in 15 μl of 100 mM Na_2HPO_4 (*transfer 2*; n = 53). During the first 30 min of contact with the subunit solution, the patch always behaved as a blank liposome, i.e., no channel activity was observed. Throughout these quiescent periods, the electrode voltage was clamped for various time intervals at several values between ±100 mV, since it seemed possible that voltage could influence incorporation or activation. If, after 90 min of immersion, no channel activity was observed, or if the seal resistance changed dramatically, the patch was discarded, and we assumed either no incorporation or a multilamellar patch.

However, we occasionally observed incorporation of the 150-kDa subunit (n = 5) marked by the appearance of unitary conductance jumps ranging from 1.9 to 8.5 pS in 100 mM Na_2HPO_4 (Fig. 4) and averaging 4.8 ± 0.151 pS (n = 80 events). The channels fluctuated between the open and closed states, with open times ranging from 24 ms to 9.1 s. MOT averaged 728 ± 184 ms (n = 80); the channel was open 8.5% of the time of observation (see DISCUSSION). No correlation was observed between the conductance and open time of a single event. The single-channel current, conductance, and the open time were independent of voltages from −60 to +100 mV. We did not observe more than one open channel at a time, which was not surprising due to the time required for the appearance of the first conductance jump after immersing the patch electrode in the subunit solution (~45 min).

Transfer of the patch containing an active open subunit to another well containing 0.1 μM amiloride (*transfer 3*; n = 3) resulted in rapid transitions between the open and blocked conductance states (see Fig. 5, *B* and *C*). After transfering the electrode tip to the amiloride vial, we never observed an event longer than 60 ms. For the three successful transfers, 184 channel fluctuations were observed over a 3.18-s period of time, and the MOT averaged 13.8 ± 0.94 ms. Patch seals were lost presumably before spontaneous channel closings occurred, since long closed times of several seconds were not observed. Thus no information was obtained for mean closed time in the presence of amiloride. We conclude that the rapid transitions were caused by the reversible binding and inhibition of an open channel by amiloride. Futhermore, if amiloride had not been present, we assume that the patch current would have been stable at the open level during this time (i.e., P_o = 1). However, during these periods of amiloride exposure, the channel was blocked 19.2% of the time (P_o = 0.81). These data would support the idea that amiloride is blocking ion movement mediated by the 150-kDa subunit. The characteristics of the isolated 150-kDa subunit of the Na^+ channel protein are similar to the behavior of the native channel studied in cell-attached patches in A6 cells (5) and toad urinary bladder (6) as well as in planar lipid bilayer membranes (25).

DISCUSSION

Characterization of the structure and function of the apical epithelial Na^+ channel is essential to understanding the physiological mechanisms of cellular salt and water movements. It is clear that the apical Na^+ channel plays a pivotal role in regulating intracellular Na^+ concentration and, consequently, the rate of transepithelial Na^+ transport. Like the channels of excitable tissues, the epithelial Na^+ channel consists of several subunits (5 or 6), and channel activity is modulated by a variety of

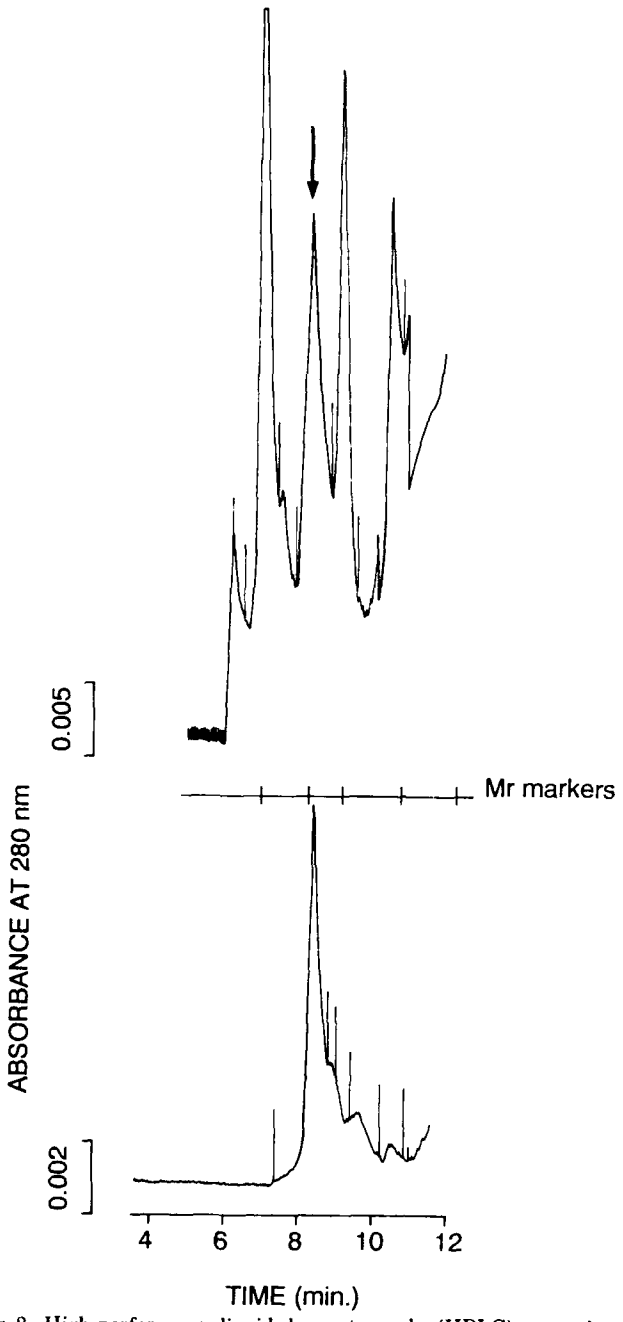

Fig. 2. High-performance liquid chromatography (HPLC) separation of Na^+ channel subunits. *Top trace*: Zorbax GF250 gel filtration HPLC separation of dithiothreitol-reduced bovine papillae Na^+ channels. Peaks were collected individually as indicated by vertical marks on trace. *Bottom trace*: rechromatography of peak eluted at 8.5 min (cf. arrow, *top trace*) in previous separation [relative molecular mass (M_r) ~150 kDa]. Elution buffer was 100 mM Na_2HPO_4, pH 7.0, at flow rate of 1 ml/min. Molecular weight markers (Bio-Rad) are denoted by hash marks between the 2 traces that (from *left* to *right*) were thyroglobulin (670,000), γ-globulin (158,000), ovalbumin (44,000), myoglobin (17,000), and vitamin B-12 (1,350).

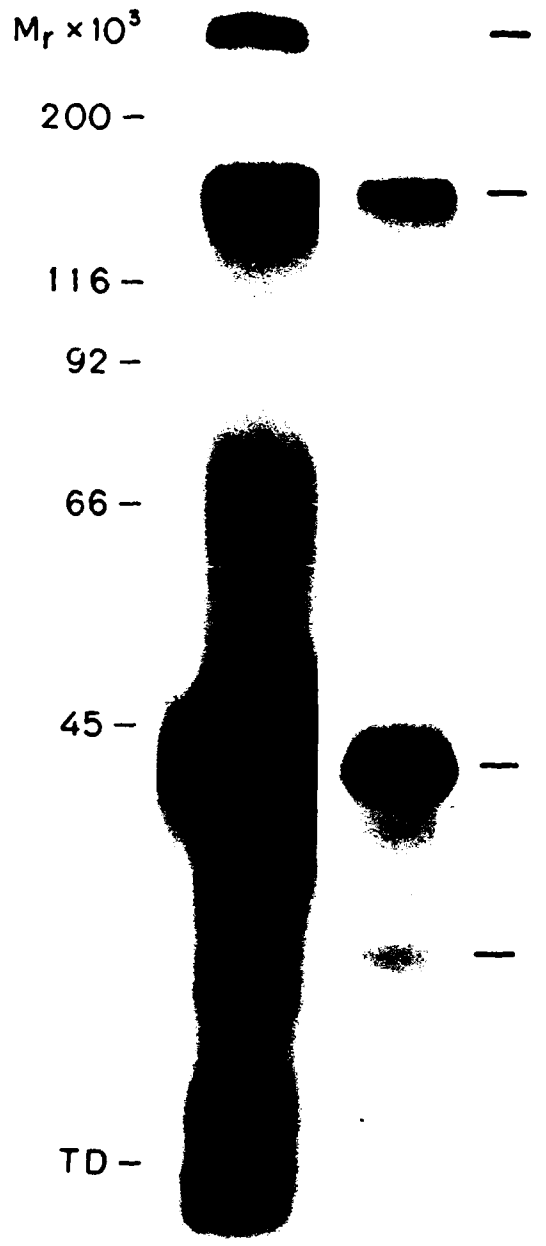

Fig. 3. Autoradiography of SDS-polyacrylamide gel electrophoresis gel of peaks collected at 8.5 min in each of HPLC runs shown in Fig. 2. *Left* and *right lanes* correspond to *top* and *bottom traces* of HPLC profiles, respectively. Films were exposed to gels for 72 h (160,000 counts/min and 10 ng protein per lane). M_r marker positions determined from Coomassie Blue-stained gels are given at *left*. TD, position of tracking dye. Marks on *right* (from *top* to *bottom*): top of gel, 152-, 40-, and 31-kDa polypeptides.

hormones and other intracellular factors and second messengers (for a review see Ref. 21). Although it was possible that several of the subunits could have been required for channel activity, it also seemed plausible that a single subunit could conduct Na^+ ions with the others serving a regulatory role. Because the 150-kDa HPLC fraction yielded channel activity, we attribute both the Na^+ transport and blocker binding properties to the 150-kDa subunit of the Na^+ channel (see below). Although it is clear that other polypeptides are present in our sample (see Fig. 3, *right lane*), we must emphasize that there is no biochemical assay as sensitive as the electrical measurements of a bilayer membrane, since the incorporation of one channel molecule may be sufficient to induce current fluctuations. Clearly, even a single band on a gel would

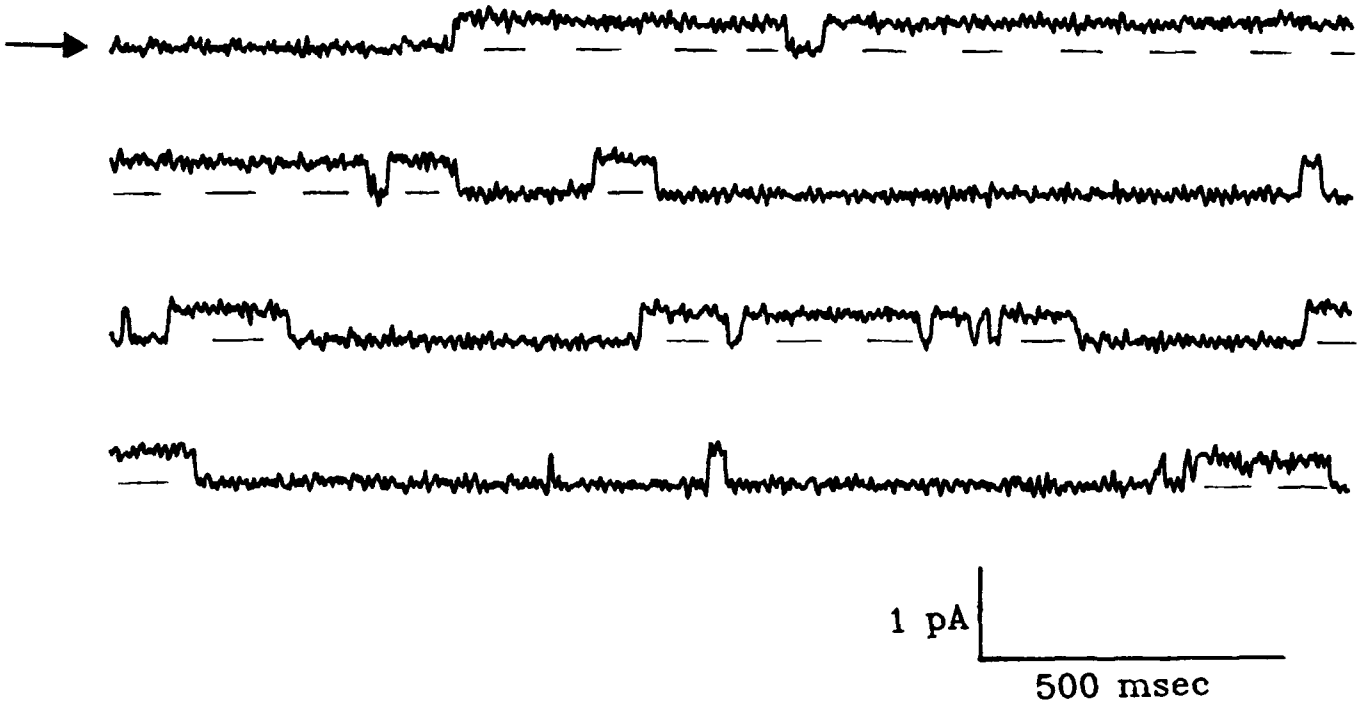

Fig. 4. Spontaneous activity recorded from excised liposome patch dipped in solution of 100 mM Na_2HPO_4 containing 150-kDa subunit. Single-channel conductance was 3.2 pS; $V_{pipette}$ = +100 mV. Arrow and dashed lines indicate current measured from quiescent membrane (closed level).

not represent irrefutable evidence that the sample consisted of pure 150-kDa subunit. Consequently, the certainty of our conclusion that the single-channel activity from this fraction represents solely the activity of the 150-kDa subunit can only be proven after cloning.

Current through the 150-kDa subunit was carried by cations. Given our low success rate when transferring the pipette between solutions, we did not measure the selectivity of Na^+ over K^+, but amiloride block of the native Na^+ channel often is associated with high Na^+ selectivity, albeit with various ratios (5, 6, 18, 25). Conversely, the lack of amiloride sensitivity often is indicative of nonselective cation channels (6, 9). However, two exceptions to this behavior have been described. Amiloride-sensitive conductances found in the apical membrane of rat inner medullary collecting duct (13) and in rabbit cortical collecting tubule primary cell cultures (15) are not selective for Na^+. In those studies, the MOT of the channels ranged from ~50 to 100 ms, and the conductance ranged from 9 to 26 pS. In the present studies, only 27 of 80 openings were <100 ms, and 13 of the events were 900 ms or longer (MOT = 3,439 ± 793 ms). In addition, the observed 4.8 pS conductance of the subunit in the present study is consistent with the high Na^+ selectivity, low-conductance, amiloride-sensitive channel described previously for other high-resistance epithelia (3, 6, 18).

At this juncture, it is premature to speculate on the mechanism of amiloride inhibition of the Na^+ channel, but binding of the inhibitor to the conducting moiety would certainly permit even the most simplistic models, such as plugging the mouth of the channel, without any direct conformational modifications of the transport protein. Although the subunit single-channel conductance and MOT were similar to the whole protein, we found that the concentration of amiloride used in the present study (0.1 μM) yielded at most a 20% inhibition of subunit current. This concentration represents the inhibitory constant (K_i) for amiloride in membrane vesicles as well as whole channel protein studied in planar bilayers (24, 25). Thus it would appear that the other subunits may modulate association of amiloride with the 150-kDa subunit. Indeed, it was surprising to find such similar single-channel properties, given the vastly different conditions and environment of our studies compared with physiological conditions.

Support for our conclusion that both the Na^+ transport and blocker binding properties pertain to the 150-kDa subunit of the epithelial Na^+ channel is provided by recent studies of *Xenopus* oocytes that were injected with fractionated poly(A)$^+$RNA from A6 cultured kidney cells (7, 17). Like the bovine papillae membranes used in the present study, Na^+ transport in A6 cells is blocked by amiloride with high affinity (24), and the Na^+ channel protein consists of subunits with molecular masses of 315, 150, 95, 71, 55, and 35 kDa (2). The injected oocytes expressed functional Na^+ channels that correlated with the translation of polypeptides with a molecular mass of 150 kDa or less and was independent of the translation of the 315-kDa subunit.

The observation that the 150-kDa subunit expresses a Na^+ conductance in an artificial lipid environment does not preclude participation of the other subunits in the overall function of the whole channel in situ (i.e., under physiological conditions) and during hormonal challenges. Indeed, the 315-kDa subunit of the Na^+ channel from cultured A6 kidney cells is phosphorylated by an adenosine 3′,5′-cyclic monophosphate-dependent protein

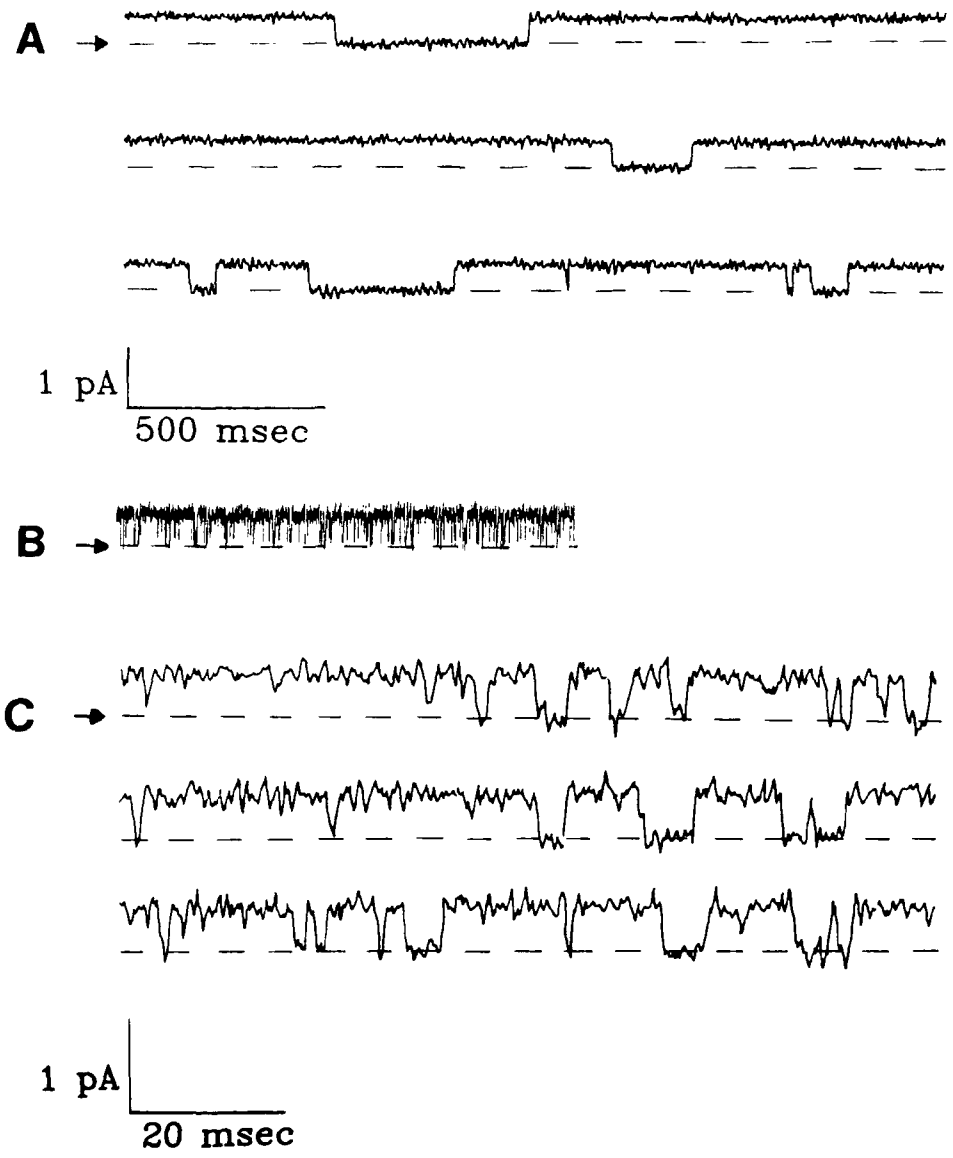

Fig. 5. Effect of 0.1 μM amiloride on single-channel activity of 150-kDa subunit. *A*: control; single-channel conductance, 7 pS; $V_{pipette}$ = +60 mV. *B*: amiloride. After obtaining single-channel activity, patch electrode was moved to well containing amiloride (*transfer 3*). Single-channel conductance, 6.3 pS; $V_{pipette}$ = +80 mV. *C*: amiloride. Small portion of trace shown in *B*. Time scale is expanded 25 times relative to traces shown in *A* and *B*. Arrows and dashed lines indicate closed level.

kinase A after stimulating Na^+ transport with vasopressin (26). Attempts to incorporate this subunit into a patched FTL membrane in the present study failed to yield channel activity (after *transfer 2*; $n = 3$). However, our purpose was not to investigate extensively the properties of each subunit, since the lack of single-channel activity may not be meaningful. Similar to our findings, Singer et al. (20) recently have shown that only a single subunit (α_1) of the type L calcium channel is necessary for Ca^{2+} movement, but the other subunits are required for modulation of the channel function, including agonist potentiation, activation, and inactivation times, voltage dependence, and tissue specificity. Clearly, elucidation of the contribution of each subunit to the global function of the native Na^+ channel, as well as the interactions between those subunits, awaits cloning of the gene(s) encoding this complex epithelial protein. Indeed, the present observations support the feasibility of using expression cloning to isolate cDNA clones that encode the epithelial Na^+ channel.

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